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CHARACTERIZATION OF A VIRUS ISOLATED FROM JAPANESE EELS (*ANGUILLA JAPONICA*) WITH NEPHROBLASTOMA

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Yoichiro Ueno, Shiu-Nan Chen, Guang-Hsiung Kou, Ronald P. Hedrick and John L. Fryer (1984) Characterization of a virus isolated from Japanese eels (*Anguilla japonica*) with nephroblastoma. *Bull. Inst. Zool., Academia Sinica* **23**(1): 47-55. The physical and biological properties of a virus isolated from Japanese eels (*Anguilla japonica*) with nephroblastoma were examined. The virus was icosahedral with a diameter of 75 nm. Infectivity was retained following with chloroform or ether treatment. The virus was stable at pH values between 2.2 and 9.0 and labile to temperature above 70°C. Neutralization and fluorescent antibody tests demonstrated a close relationship between this virus and eel virus European (EVE). Eels, tilapia (*Oreochromis aureus* × *Oreochromis niloticus*), and carp fry (*Cyprinus carpio*) injected intraperioneally with virus grown in eel ovary (EO-2) cell line experienced greater mortalities than control groups which received no virus. Mortality was greatest among tilapia held at 10-16°C.

Reports appearing during the past ten years describe several viurses isolated from eels. These include EV-1 (Koops *et al.*, 1970), EV-2 (Nagabayashi and Wolf, 1979; McAllister *et al.*, 1977), eels virus European (EVE) (Sano, 1976), EVA (Sano, 1976), and EVEX (Sano *et al.*, 1977). Both EVA and EVEX are rhabdoviruses and shared certain antigenic characteristics (Nishimura *et al.*, 1981). The viurses EV-1, EV-2 and EVE possess unique morphology and appear antigenicity unrelated. EV-1 may be a papivirus (Schwartz-Pfitzner, 1976; Pfitzner and Schubert, 1969) while EV-2 is apparently a member of the orthomyxovirus group (Nagabayashi and Wolf, 1979). EVE belongs to those viruses tentatively classified as Birnaviruses and is more closely related serologically and biochemically to the Ab strain of infectious pancreatic necrosis viurs (IPNV) (Okamoto *et al.*, 1983; Hedrick *et al.*, 1983).

McAllister *et al.* (1977) reported EV-2 to be pathogenic in American eels (*Anguilla*

rostrata). Fifity percent of the eels injected intraperitoneally with virus died while only 4% of the controls died. However, the virus could be reisolated from only 25% of the dead fish.

Initially, EVE was demonstrated to be pathogenic for Japanese eels (*Anguilla japonica*) after intraperitoneal administration of virus (Sano *et al.*, 1981). Subsequence infectivity trials were unsuccessful in inducing mortality causing the authors to conclude that propagation of the virus in RTG-2 cells derived from rainbow trout (*Salmo gairdneri*) instead of homologous eel cells may have resulted in attenuation (Sano *et al.*, 1981)

This paper characterizes the physical and biological properties of a virus isolated from eels with nephroblastoma. In addition, the pathogenic properties of the virus in selected warm-water fishes were tested following cultivation in the eel ovary cell line (EO-2) (Chen and Kou, 1981). The homologous line, EO-2 was used to alleviate possible problems due to attenuation of the virus during passages in heterogeneous cell lines.

MATERIALS AND METHODS

Cell lines

The RTG-2 (Wolf and Quimby, 1962) and FHM (Gravell and Malsberger, 1965) cell lines derived from rainbow trout gonad (*Salmo gairdneri*) and fathead minnow (*Promephales pimelas*) respectively were grown in Eagle's minimal essential medium (MEM, Flow Lab.) supplemented with 10% fetal calf serum (MEM-10), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml fungizone (Grand Island Biological Co.). The cells were incubated at 10-20°C.

The EK-1 and EO-2 cell lines derived from the Japanese eel (*A. japonica*) kidney and ovary tissues respectively were propagated in Leibovitz's medium (Flow Lab.) supplemented with the same concentrations of serum and antibiotics as was MEM. The cells were incubated at 31°C for routine cultivation.

Viruses

Virus used in this study was isolated from five eels with nephroblastoma collected in March, May and August 1981. The virus was isolated from internal organs. The culture fluids from RTG-2 and EK-1 cell lines showing cytopathic effect were subcultured at 1:100 dilutions on fresh cell cultures. Following five subcultures the suspension was frozen at −70°C and held as stock virus. Eel virus European (EVE) was obtained from Dr. Sano, Department of Aquaculture, Laboratory of Fish Pathology, Tokyo University of Fisheries, Tokyo, Japan.

Electron microscopy

Tissues excised directly from nephroblastomas or infected EK-1 cells were examined for the presence of viral particles. EK-1 cells infected with virus suspensions were harvasted three days after inoculation (incubation temperature, 18°C) by centrifugation at 1,000×g for 10 min. The cell pellet from EK-1 cells and nephroblastoma tissue were fixed with 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2). Tissues were postfixed in 1% OsO_4 for 2 h and following dehydration embedded in Epon. Thin sections stained with uranyl acetate lead citrate were observed using a JEOL 100 CX electron microscope.

Virus titration

The concentration of virus in infected cell suspensions were estimated by tissue culture infective dose 50% ($TCID_{50}$/ml) analysis using EO-2 cells in 96-well plates (Linbro) as described by Chen and Kou (1981).

Effect of pH and organic solvents on viral activity

The effect of pH on the infectivity of the virus was examined following incubation in phosphate (pH 2.2 to 8.0) and carbonate (9.0 to 11.0) buffers. One volume of virus was combined with nine volumes of buffer. Following 3 h incubation at 20°C, the pH was adjusted to 7.4 and then the concentration of the virus determined by $TCID_{50}$/ml analysis.

The method described by Andrewes and Horstmann (1949) was used to determine the effect of ether on viral infectivity. Four volumes of the viral solution were mixed with one volume of ether in a sealed tube and incubated at 4°C for 24 h. The tube was shaken occasionally during incubation. Viral infectivity was then determined by $TCID_{50}$/ml analysis.

The effect of chloroform on virus activity was determined using the method of Fedman and Wang (1961). Briefly, 4 ml of the viral solution were mixed with 2 ml of chloroform in a sealed tube and shaken vigorously for 10 min. The concentration of virus in the upper phase was then titrated. In both the ether and chloroform sensitivity experiments, virus was suspended in 0.85% NaCl and held under the same conditions but without lipid solvents treatment were used as controls.

Effect of temperature on viral stability

The stability of virus in L-15 with 10% fetal calf serum (pH 7.2) was tested at temperatures between 50–70°C. An aliquot of virus suspension at 0.1 ml was withdrawn at 10 min intervals and titrated. A virus suspension held at room temperature was as a control.

Fluorescent antibody and neutralization tests

The indirect fluorescent antibody assay and serum neutralization tests were used to identify the virus from eels with nephroblastoma. The procedures for staining virus-infected EK-1 cells were similar to those described by Tu *et al.* (1974). Coverslips culures of EK-1 cells were fixed in cold acetone for 10 min and then air dried 12 h following infection with virus. The cells were then exposed to rabbit anti-VR 299 IPNV or anti-EVE serum prepared as described by Okamoto *et al.* (1983). After 30 min at room temperature (25°C) the cells were rinsed three times in phosphate-buffered saline (PBS pH 7.4). Goat anti-rabbit serum labelled with fluorescent isothiocyanate (BBL) was then added and incubation continued for an additional 30 min. The cells were then rinsed three times with PBS, mounted on slides with buffered glycerol (pH 7.5) and observed with an Olympus Vanox microscope equiped with 200 W mercury lamp.

Neutralization tests with rabbit anti-EVE serum were also used to confirm the identity of the virus from eels. The methods for this test were the same as those described by McDaniel (1979).

Infectivity of virus in selected warm water fish

Japanese eels, common carp (*Cyprinus carpio*) and hybrid tilapia (*Oreochromis aureus*× *Oreochromis niloticus*) juveniles were inoculated with the virus to determine its pathogenic properties. The fish weighed from 5–25 gm each. Experimental groups received an intraperitoneal injection of 0.1 ml $10^{8.3}$ $TCID_{58}$/ml per 5 gm of body weight. Control fish received an equal volume of L-15 containing 5% fetal calf serum without virus. All groups of fish were held in 20 l aquaria at 20–25°C. In addition, a second test with tilapia was conducted at 10–16°C. Mortalities from all groups were recorded and examined for the presence of virus.

RESULTS

Primary isolation of virus

All diseased eels showed whitish, swollen and solid kidneys (Fig. 1). Spleen, kidney, liver and gonad homogenates made from these eels revealed the presence of a virus that induced CPE in both RTG-2 and EK-1 cells after 6–8 days incubation at 18°C. Subsequent subcultures on EK-1 cells revealed the appearance of CPE in 2–4 days.

Electron microscopy

Virus was not observed in tissues taken from the nephroblastoma. Icosahedral shaped virus particles were, however, observed in infected EK-1 cells (Fig. 2). The virus had the typical morphology of the Birnavirus group. The diameter of the particles was approximately 75 nm.

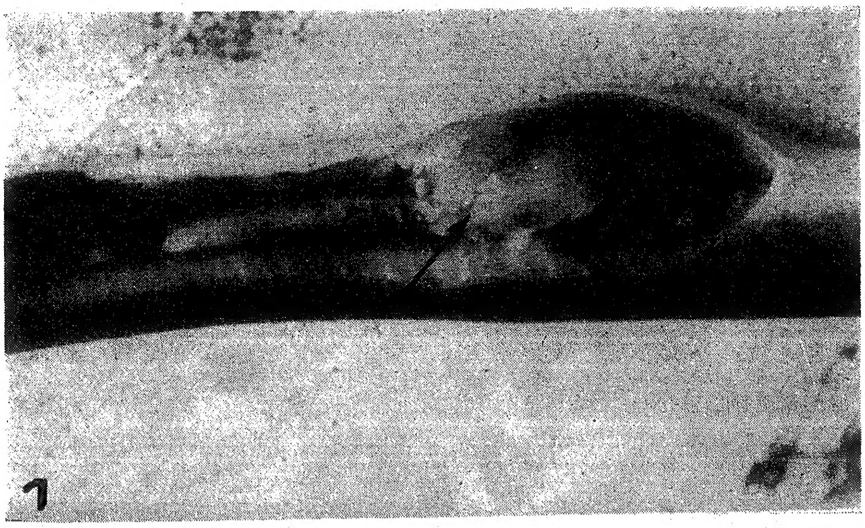

Fig. 1. The experimental eel with nephroblastoma. Note the whitish, swollen and solid kidney (arrow).

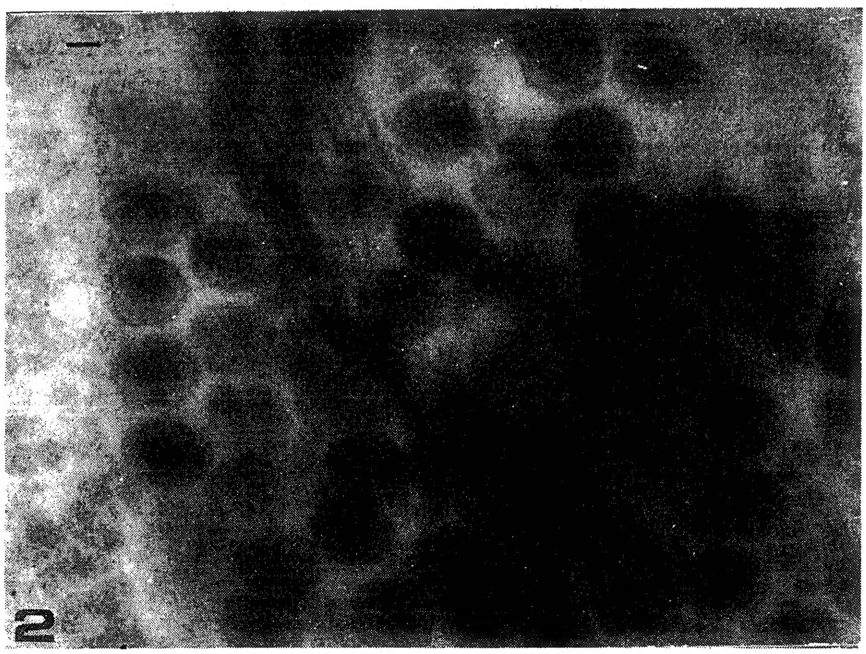

Fig. 2. Isolated virus particle in EK-1 cells. Note the iscosahedral particle of approximately 75 nm in diameter (Bar=20 nm).

Fig. 3. Photomicrograph of indirect immunofluorescent stained EK-1 cells infected with isolated virus using anti-EVE rabbit serum and FITC labelled anti-rabbit IgG (300×).

TABLE 1.
The effect of pH of culture medium on the activity of isolated virus

pH of Culture Medium	Viral Activity
2.2	8.45±0.15
3.0	8.15±0.10
4.0	8.32±0.07
5.0	8.30±0.09
6.0	8.41±0.30
7.0	8.16±0.21
8.0	8.10±0.15
9.0	8.29±0.41
10.0	7.09±0.01
11.0	6.22±0.01

The viral activity was titrated by using EO-2 cells at 18°C as described in the section of Materials and Methods and expressed as $\log_{10}$ $TCID_{50}$/ml.
Each test was performed at least in duplicate.

TABLE 2.
The effect of organic solvents on the activity of isolated virus

	Ether	Chloroform	Control
Activity in $\log_{10}$ $TCID_{50}$/ml	9.21±0.07	9.14±0.12	9.05±0.23

The viral activity was titrated using EO-2 cells at 18°C as described in the section of Materials and Methods.
Each test was performed at least in duplicate.

Effect of pH, organic solvents and temperature on viral infectivity

The infectivity of the virus following 3 h incubation at selected pH values was determined (Table 1). The results presented in

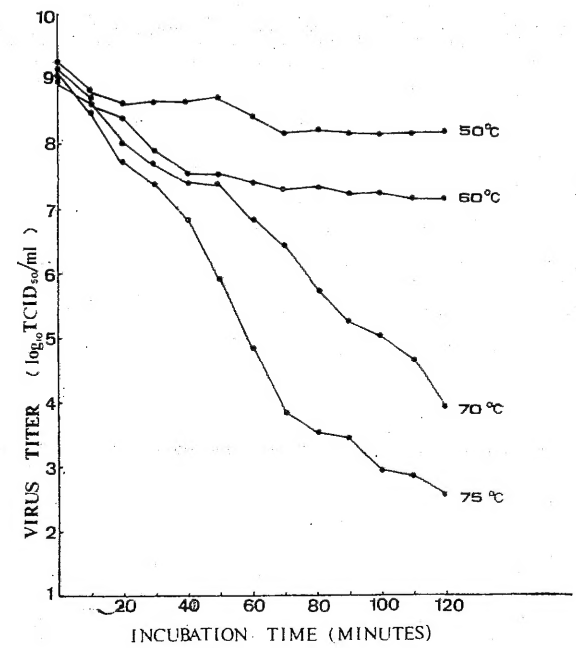

Fig. 4. Decline of activity of isolated virus at various incubation temperatures.

Table 1 showed the virus lost little infectivity between pH 2.2 and 9.0. Infectivity was decreased at pH 10.0 and 11.0.

The virus was resistant to chloroform and ether treatment (Table 2) indicating no essential virus associated lipids are present.

The stability of the virus after 2 h at 50, 60, 70 and 75°C was demonstrated (Fig. 4). A steady decline in infectivity from the original

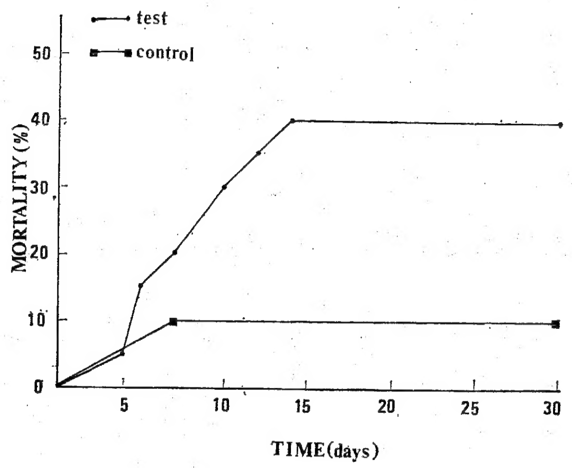

Fig. 5. Cumulative mortality curve of eel (*Anguilla japonica*) infected with isolated virus by an intaperitoneal injection and maintained in a water temperature between 20-25°C.

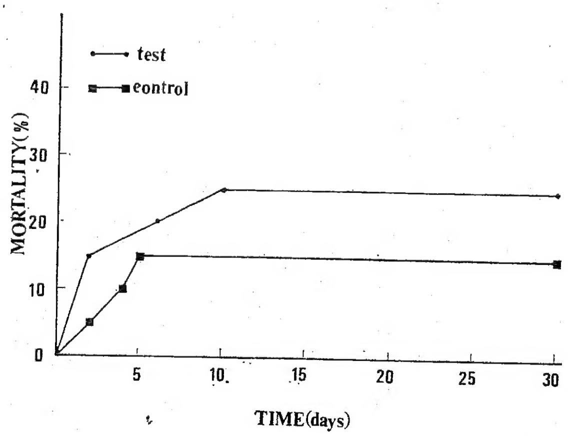

Fig. 6. Cumulative mortality curve of carp (*Cypurinus carpio*) infected with isolated virus by an intraperitoneal injection and maintained in a water temperature between 20-25°C.

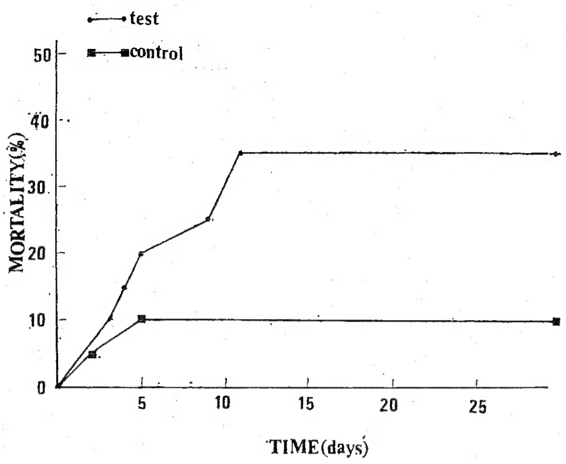

Fig. 7. Cumulative mortality curve of hybrid tilapia (*Oreochromis aureus*×*Oreochromis niloticus*) infected with isolated virus by an intraperitoneal injection and maintained in a water temperature between 20-25°C.

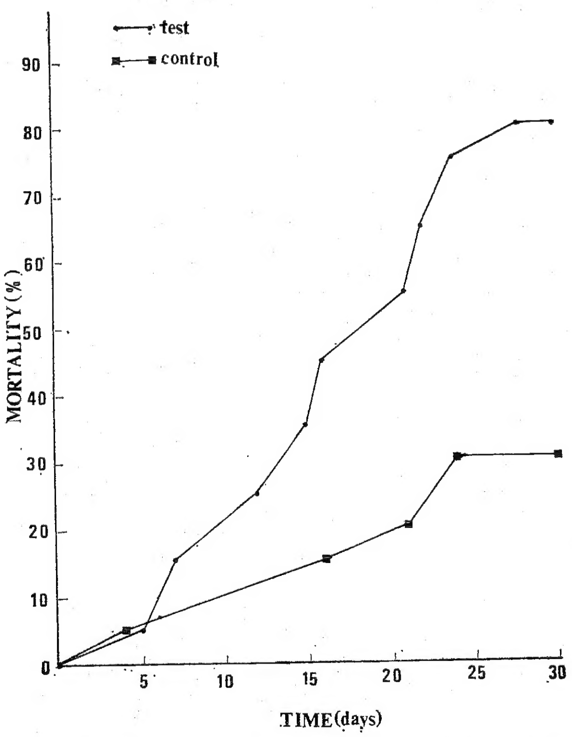

Fig. 8. Cumulative mortality curve of hybrid tilapia (*Oreochromis aureus*×*Oreochromis niloticus*) infected with isolated virus by an intraperitoneal injection and maintained in a water temperature between 10-16°C.

inoculum was observed as the temperature of incubation increased.

Fluorescent antibody and serum neutralization tests

Fluorescent antibody assays with anti-EVE serum indicated that the virus from eels with nephroblastoma was similar to EVE. Cells infected with the virus stained the same intensity as those infected with EVE (Fig. 3). Subsequent neutralization tests confirmed a close serological relationship between the two viruses.

Infectivity of virus in selected warm water fishes

The mortalities experienced by eel, carp and tilapia injected with virus were compared (Fig. 5–7). Deaths among inoculated eels 40%, 25% for carp and 35% for tilapia. Mortality among control groups was only 10–15%. By lowering the water temperature to 10–16°C, deaths among tilapia inoculated with virus increased to 80% while uninoculated controls experienced a 30% loss (Fig. 8). Virus was reisolated from each death fish in the experimental groups. No virus was detected in fish from the uninoculated groups.

DISCUSSION

This study showed that virus could be isolated from several tissues (spleen, liver, kidney and gonad) of eels with nephroblastoma. The inability to detect virus by EM observation of nephroblastoma tissues may be due to insufficient concentrations of viral particles.

The virus isolated possessed several properties similar to those of EVE reported by Sano *et al.* (1981) such as particle morphology and size, tolerance to organic solvents, stability at pH 2.2 to 9.0 and higher incubation temperatures (50–75°C). The results of fluorescent antibody and neutralization tests confirmed that the virus has a close antigenic relationship to EVE.

To determine the pathogenicity tests of EVE, Sano *et al.* (1981) reported that the virus killed Japanese eels but not rainbow trout fry. Their data also indicated that the pathogenicity of EVE against *A. japonica* decreased after several passages in RTG-2 cells. In the present study, EVE subcultured in EO-2 cells was used and this virus induced mortality in tilapia, carp and Japanese eel elevers at maintenance water temperatures of 20–25°C. This observation further confirm our previous position that virus originating from homologous cell cultures is probably superior to that from heterogous cell cultures in testing the pathogenicity of viruses in fish (Chen and Kou, 1981).

The present study also showed that the mortality of virus-infected tilapia was approximately 80% at water temperatures of 10–16°C, which is significantly different from those obtained at the water temperatures of 20–25°C. Sano *et al.* (1981) suspected that EVE was the primary cause of branchionephritis (Egusa, 1970) which had occurred annually during the winter season among eels reared in Japan. Branchionephritis has also occurred frequently among cultured eels in winter months causing serious mortality. These observations, together with our experiments, suggest that water temperature and cell line used to propagate the virus are important factor(s) affecting the course of infection in experimentally inoculated fish.

Tumors found in various fish suspected to be caused by viruses have been reported in northern pike (Muleahy, 1976), muskellunge (Sonstegard, 1976), guppy (Wessing and Bargen, 1959) and eel (McAllister *et al.*, 1977). However, viruses have not been demonstrated to be the cause of these tumors. Only one successful trial in the viral inducing tumor of fish has been reported by Kimura *et al.* (1981). They isolated an oncogenic herpes virus, Oncorhynchus masou virus (OMV), from the ovarian fluids of normal appearing adult laldlocked masou salmon (*Oncorhynchus masou*). They showed that epithelial tumors developed following injection of OMV into young chum salmon. In the present study, although EVE was isolated from eels with nephroblastoma, no tumors were induced following injection of

virus. Therefore, causes of nephroblastoma in eels remain under investigation.

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從罹患腎腫瘤白鰻 (*Anguilla japonica*) 所分離出的病毒特性研究

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R. P. HEDRICK AND J. L. FRYER

本研究擬探討從罹患腎腫瘤的鰻魚（*Anguilla japonica*）所分離出病毒之物理及化學特性。此病毒爲直徑約 75 nm 之二十面體，對哥羅芳（Chloroform）及乙醚（Ether）具有抵抗力。在培養溶液之酸鹼度 2.2～9.0 之間，病毒活性穩定，但於溫度 70°C 以上則其活性會降低。由中和反應及螢光抗體試驗證實本病毒之血清型與 EVE 很接近。當以鰻魚、鯉魚及吳郭魚進行病毒之腹腔注射時，則實驗組（經病毒注射者）比控制組之死亡率高。當水溫控制於 10～16°C 時，注射病毒之吳郭魚死亡率最高。